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PREPARATION AND PROPERTIES OF AN *o*-DIPHENOL: O_2 OXIDOREDUCTASE FROM COCOA HUSK

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Abstract—A particulate preparation from cocoa husk which shows *o*-diphenol: O_2 oxidoreductase activity contains a copper protein moiety linked to a partially formed insoluble polyphenol polymer. The particles are easily stained with osmium tetroxide for electron microscopy and show marked *o*-diphenol-polymerisation properties when incubated with substrate. The activity and kinetic parameters of the particles against a number of substrates and inhibitors have been determined.

INTRODUCTION

o-Diphenol: O_2 oxidoreductase (DPO, EC 1.14.18.1) is the enzyme responsible for the oxidation of *o*-diphenols to *o*-quinones by molecular oxygen in various plants. The *o*-quinones produced may or may not polymerize and give rise to numerous pigments. The enzyme has been purified and characterized from many fruits, including apple [1], banana [2] and olives [3]. In addition to the production of pigments during the normal ripening process of these fruits the enzyme is responsible for the browning reaction when the fruit is cut or damaged. This reaction is thought to be a protection mechanism as the quinones produced have been found to partially inactivate certain viruses by covalent modification [4], and in any event form an isoluble barrier over the damaged area. *Phytophthora palmivora* (Butl.) Butl., a fungus which is the causative agent of Black Pod disease in the cocoa (*Theobroma cacao* L.) pod husk, produces a distinctive brown discoloration on the husk. This is due to the husk DPO, decompartmentalized by fungal pectinase, acting on husk diphenols [5]. There has been only a little work on cocoa DPO in the past and this has been concerned with the leaf enzyme [6].

This work was undertaken in order to determine the mode of action of cocoa pod husk DPO in the hope that an increase in the knowledge of this enzyme might contribute to an eradication program for Black Pod disease.

RESULTS

The brown precipitate produced by differential centrifugation of the resuspended acetone-dried powder had an average particle diameter of 200 ± 40 nm giving an approximate particle MW of 3×10^9, assuming an estimated density of 1.4 g/cm^3. The particles were heavily stained for electron microscopy and they could be easily distinguished, attached to the cell walls in both ripe pods and cherelles. In addition the ripe pods contained some heavily stained larger sheet-like structures (*ca* 1 μm/long). There particles formed fibres (polymers) on incubation with the catechol substrate. These fibres (40 × 850 nm) stained lightly on the outside only.

Table 1. Michaelis constant (K_m), substrate inhibition constant ($K_{m'}$) and maximum velocity of DPO substrates

Substrate	Max (nm)	K_m (mM)	$K_{m'}$ (M^{-1})	V_{max}, A/mM/min relative to catechol (−1.0)
Catechol(ripe pod)	400	14.4	30	1·0
Catechol(cherelles)	400	14.3	nd	1.0
Quinol	400	79		0.2
3-Methyl catechol	400	20	18	0.4
4-Methyl catechol	400	2.5		0.5
Epicatechin	430	1.7		0.7
Catechin	400	2.1		0.3
		34		3.3
Pyrogallol	300	1.7		0.5
L-Adrenalin	480	13.7		0.2
Gallic acid		nar		
p-Cresol		nar		

nd = not determined.
nar = no apparent reaction

The chemical analysis of the particles was determined as protein 67.2%, carbohydrate 0.1% copper 0.42% with a phenolic residue which was insoluble in all the organic solvents tried (e.g. 5.5 N refluxing HCl, ether, acetone and ethanol). From the copper and protein analysis, the ratio of protein to copper in the enzyme particle was calculated to be MW 10000/copper atom. Thus there were about 10^5 copper atoms/DPO particle.

Table 2. Inhibition constants with DPO

Substance	Inhibition constant K_i (M)	$K_{i'}$ (M)
p-Cresol	—	1.2×10^{-2}
L-Cysteine	2.5×10^{-5}	—
Quinol	1.6×10^{-5}	—
Cyanide	1.4×10^{-2}	1.2×10^{-2}

The IR analysis gave peaks characteristic of proteins (3400–3300 cm^{-1}, 1650 cm^{-1}), aromatic rings (3000 cm^{-1}, 1480 cm^{-1}) and ketones (1680 cm^{-1}), indicating that the DPO particles contained a mixture of protein and partially oxidized phenolic residues.

The cocoa husk DPO kinetic constants are shown in Tables 1 and 2. The initial rate of chromogen formation was linear with respect to DPO concentration up to 1% protein content. The variation of DPO activity (V_{max}) with pH using catechol as substrate, gave a fairly symmetrical peak centred on a maximum at pH 6.2, similar to that expected if the rate depended on general acid-base catalysis involving two histidine residues both with a pK_a of *ca* 6.2.

DISCUSSION

The enzyme as prepared from the ripe husk or the cherelles appeared identical, by comparison of the K_ms obtained. Both are derived from a particular, deep staining, fraction attached to the cell wall. DPO from other sources have also been found to be particulate [7] although this is by no means a general occurrence.

The cherelle DPO preparation differed from the precipitable ripe pod DPO in so far as it was not precipitated on centrifugation (20000 *g*, 30 min) but gave a greenish opaque solution. It may be assumed that the difference is due to variation in the polymerized phenolic/quinone content of the particles.

All the substrates tested showed strong irreversible product inhibition. This may be due to the *o*-quinone structure produced combining with free lysine or arginine groups on the enzyme [4,8]. It should be noted that the substrates were usually in about $10^2\times$ excess over the DPO. In addition, catechol and 3-methyl catechol showed substrate inhibition at high substrate concentrations. This however was not shown by substrates substituted in the 4-position and may be due to a subsidiary diphenol binding site normally blocked by the 4-subsituted substrate initially bound.

Catechin appeared to be oxidized by two separate isoenzymes as a very distinct combination of two straight lines was obtained in the kinetic plot. Isoenzymes have been commonly found in soluble DPO [9] but this may be the first indication in particulate DPO. The behaviour of quinol is also best explained on the basis of isoenzymes, the quinol behaving both as a substrate, to one isoenzyme, and an inhibitor, to the catechol oxidizing isoenzymes (see below). The kinetic constants and chemical analysis must therefore refer to the net effect of these isoenzymes. The particles could not be solubilized for disc electrophoresis by urea (8 M), SDS (1%) or Triton X (2%) treatment and therefore the presence of isoenzymes could not be proved or disproved by the normal methods [9].

It appears that an alkyl group in the 4-position increases the binding (decreases K_m) of the substrate but has minimal effect on the V_{max}. The electron withdrawing carboxyl group in gallic acid prevents reaction, however.

All 3 types of inhibition were shown by the inhibitors chosen. L-cysteine and quinol were competitive inhibitors whereas *p*-cresol was uncompetitive and cyanide noncompetitive. These results indicate that there must be at least two sites on the enzyme, both probably containing copper; a phenolic substrate binding site and an oxygen substrate binding site. From subsequent analysis of the acidic pK_as of the substrates it was found that the phenolic binding (K_m) was independent of the acidity of this group and other factors must be important for the binding of the phenolics to the copper atoms.

It appears that the enzyme particles contain a mixture of enzyme activities. The major activity, *o*-diphenol: O_2 oxidoreductase, is specific for *o*-diphenols and is unable to oxidize *p*-cresol, and the minor activity, *p*-diphenol: O_2 oxidoreductase (formerly known as laccase) which can oxidize quinol.

EXPERIMENTAL

Materials. Ripe brown cocoa pods and green cherelles (2- to 3-month-old fruits) were obtained from the Cocoa Research Institute, Tafo or the Botanical Gardens, Legon. Reagents were Analar grade as far as possible and the catechol was recrystallized $\times 2$ from toluene.

Preparation of cocoa DPO. Ice-cold Me_2CO (800 ml) was added to *ca* 200 g of fresh-frozen ripe cocoa pod husk and homogenized rapidly at 0°. The product was rapidly air dried and stored at −20°. The Me_2CO dried powder was homogenized rapidly with 0.01 M ice-cold ascorbic acid in 0.1 M NaPi buffer (pH 6.8) and kept at 4°. Cell debris was removed by centrifugation for 10 min at 2000 *g*. The active DPO fraction was prepared, from the ripe husk, as a particulate fraction, by centrifugation at 20000 *g* at 0–4° for 30 min. In the preparation from cherelles the active DPO was found in the greenish slightly opaque soln left after this centrifugation. Neither preparation would enter the acrylamide gel for disc electrophoresis.

Assay of DPO activity. Oxidation of catechol by DPO and O_2 produced a series of chromophores. The production of the first of these was used in the assay. Catechol soln was prepared in 0.1 M NaPi: buffer pH 6 Enzyme extract (25 µl) was added to 2 ml of the catechol soln, or other substrate or substrate plus inhibitor soln, and the change in A measured with time. The standard DPO assay used 30 mM catechol, measuring the A at 400 nm.

Determination of kinetic constants. The K_m and V_{max} for the various substrates were determined, using at least 5 replicates over a range of substrate concns of *ca* 100-fold, using the Eadie–Hofstee plot [10]. Inhibition constants were determined using 3 inhibitor concns at 5 substrate concns and the data analysed by standard methods [11, 12]. Substrate inhibition constants were determined from plots of substrate concentration against the reciprocal of the reaction velocity.

Analysis. Carbohydrate was assayed using the cysteine–sulphuric acid assay [13] using glucose as standard. Protein was determined as amino acid by the ninhydrin assay [14] after hydrolysis (5.5 M HCl, 105°, 16 hr) using egg albumin as standard. Cu content was determined by atomic absorption spectroscopy after ashing 520° (25 hr) and redissolving in 1% lanthanum chloride in 1% HNO_3. IR spectroscopic analysis on the DPO particles was performed using a KBr disc. Electron microscopy was carried out at the University of Leeds using glutaraldehyde-fixed samples.

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